

REMARKS

Claims 1-5, 10, 11, 15, 25 and 29 are now pending. By this Amendment, claims 1, 4, 11, and 15 are amended; claims 6-9, 12-14, 16-24 and 26-28 are canceled; and claim 29 is added.

Applicants appreciate the courtesies shown to Applicants' representative by Examiner Baskar in the October 8, 2004 personal interview. Applicants' separate record of the substance of the interview is incorporated into the following remarks.

Entry of the amendments is proper under 37 CFR §1.116 since the amendments: (a) place the application in condition for allowance for the reasons discussed herein; (b) do not raise any new issue requiring further search and/or consideration since the amendments amplify issues previously discussed throughout prosecution; (c) satisfy a requirement of form asserted in the previous Office Action; and (d) place the application in better form for appeal, should an appeal be necessary. The amendments are necessary and were not earlier presented because they are made in response to arguments raised in the final rejection. Entry of the amendments is thus respectfully requested.

In particular, claim 1 is amended to incorporate the definition of "established in culture" in the present specification at page 3, lines 3-5. Since the phrase "established in culture" is defined such that the bacterium is obtained reproducibly and multiplies over time, amending claim 1 to incorporate this feature merely makes explicit a feature that was implicitly in claim 1. Thus, making this amendment does not require any further search or examination. Claim 4 is merely amended to clearly recite that the antigen is an isolated antigen. It is respectfully submitted that this feature was previously at least implicit in claim 4 and therefore amending the claim to recite this feature does not provide the need for further search and/or examination. In addition, claim 11 is merely amended to be in independent form. Furthermore, claim 15 is merely amended to depend from claim 11. In

addition, corresponding claim 29 is merely added to depend from claim 25. Since these claims depend from allowable claims 11 and 25, these amendments do not create the need for further search and/or examination.

Claims 1-5, 10, 11 and 25 are under examination. Claims 6-9, 12-24 and 26-28 are withdrawn from consideration. Claims 6-9, 12-14, 16-24 and 26-28 are canceled herein. In addition, claim 15 is amended herein to depend on elected claim 11. Therefore, claim 15 should be rejoined and considered in the present application.

Claims 1-5, 10, 11 and 25 are rejected under 35 U.S.C. §112, first paragraph. An appropriate Declaration of Deposit is attached hereto. Therefore, the rejection should be withdrawn.

Claims 1, 2 and 4 are rejected under 35 U.S.C. §102 over Schoedon et al. Applicants respectfully traverse the rejection.

Claim 1 is directed to a *Tropheryma whippelii* bacterium that is isolated and established in culture such that the bacterium reproducibly multiplies over time. In contrast, Schoedon teaches studies carried out on primary cultures of human blood monocytes. As discussed in the present specification at page 2, lines 19-24, such a culture cannot be used as a basis for establishing the bacterium in culture in such a way that it can be multiplied because the mean lifetime of monocytes is only 30 days, which is insufficient in view of the doubling time of the bacterium. In particular, since human blood monocytes do not multiply, the primary culture described in Schoedon cannot be used as a basis for multiplication of the bacterium. See Raoult et al., The New England Journal of Medicine, Vol. 342, No. 9, pp. 620-625 (March 2, 2000), provided with the Information Disclosure Statement (IDS) filed May 15, 2002, which indicates that the isolate described in Schoedon "could not be sub-cultured" (p. 620, col. 2). Thus, Schoedon does not teach a bacterium isolated and established in culture such that the bacterium reproducibly multiplies over time.

In addition, Schoedon is not an enabling disclosure. In particular, as described in Maiwald et al., "Cultivation of *Tropheryma whippelii* bacteria that has been established in culture such that it reproducibly multiplies over time from Cerebrospinal Fluid," Journal of Infectious Diseases, Vol. 188, pp. 801-808 (2003) (copy attached), the finding of Schoedon of the propagation of bacteria could not be confirmed in subsequent studies. Maiwald et al., p. 802, col. 1, lines 4-9, and p. 805, col. 2, lines 2-5. This was further confirmed by one of the authors of Schoedon, Martin Altwegg, who indicated in a 1999 article by Hinrikson et al., International Journal of Systematic Bacteriology, Vol. 47, pp. 1701-1706, which was submitted with the IDS filed May 15, 2002, that the relationship between clinical manifestations of Whipple's disease and different infecting strains of *Tropheryma whippelii* has not been studied "mainly because of the absence of reliable cultures (Schoedon et al., 1997)." Hinrikson et al., p. 1705, col. 1, discussion. In fact, the summary of this article on p. 1701 refers to *Tropheryma whippelii* as "the uncultivated causative agent of Whipple's disease" (emphasis added).

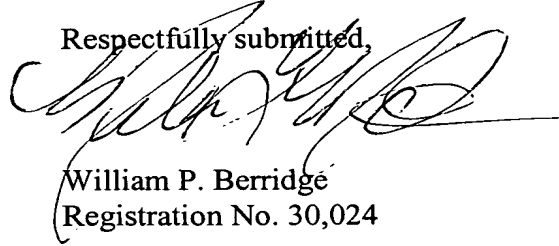
For at least these reasons, Schoedon does not teach the invention of claim 1, or claim 2 that depends from claim 1.

Claim 4 is directed to an isolated antigen of the bacterium according to claim 1. Schoedon does not teach an isolated antigen of the *Tropheryma whippelii*. Therefore, the rejection of claim 4 should also be withdrawn.

In view of the foregoing, it is respectfully submitted that this application is in condition for allowance. Favorable reconsideration and prompt allowance of claims 1-5, 10, 11, 15, 25 and 29 are earnestly solicited.

Should the Examiner believe that anything further would be desirable in order to place this application in even better condition for allowance, the Examiner is invited to contact the undersigned at the telephone number set forth below.

Respectfully submitted,



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Attachments:

Declaration of Deposit
Maiwald Article

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Cultivation of *Tropheryma whipplei* from Cerebrospinal Fluid

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(See the editorial by Scheld on pages 797–800.)

Whipple disease (WD) is a systemic disorder caused by the bacterium *Tropheryma whipplei*. Since the recognition of a bacterial etiology in 1961, many attempts have been made to cultivate this bacterium in vitro. It was eventually isolated, in 2000, from an infected heart valve, in coculture with human fibroblasts. Here we report the isolation of 2 new strains of *T. whipplei* from cerebrospinal fluid (CSF) of 2 patients with intestinal WD but no neurological signs or symptoms. One culture-positive specimen was obtained before treatment; the other was obtained 12 months after discontinuation of therapy, at a time of intestinal remission. In both cases, 15 passages of the cultures were completed over 17 months. Bacterial growth was measured by quantitative polymerase chain reaction, which suggested a generation time of 4 days. Staining with YO-PRO nucleic-acid dye showed characteristic rod-shaped bacteria arranged in chains. Fluorescent in situ hybridization with a *T. whipplei*-specific oligonucleotide probe, a broad-range bacterial probe, and a nonspecific nucleic-acid stain indicated that all visible bacteria were *T. whipplei*. Scanning electron microscopy and transmission electron microscopy showed both intracellular and extracellular bacteria. This first isolation of *T. whipplei* from CSF provides clear evidence of viable bacteria in the central nervous system in individuals with WD, even after prolonged antibiotic therapy.

In 1907, George H. Whipple described the postmortem examination of a patient who had died of a chronic disease presenting with arthritis, fever, weight loss, and cough [1]. He observed deposits of fat and fatty acids in the intestinal mucosa and mesenteric lymph nodes and named the disease “intestinal lipodystrophy.” Whipple also observed small bacteria in silver-stained sections of a mesenteric lymph node, but he did not interpret this finding as causally related to the disease. Subsequent

reports characterized Whipple disease (WD) as a rare, chronic, systemic disease, involving predominantly the intestinal tract but also a variety of other organs, especially the central nervous system (CNS) [2]. The etiology remained unclear for >40 years, until a bacterial cause was suggested by 2 observations: (1) a 1952 report of successful antibiotic treatment [3], and (2) the 1961 detection, by electron microscopy, of numerous, small, uniform bacteria in affected tissues [4, 5]. Both types of observations were subsequently confirmed and extended by many others.

Numerous attempts have been made to cultivate the WD bacterium in the laboratory, but they have either failed or yielded results that proved erroneous [2]. *Streptococcus* species, *Corynebacterium* species, and *Haemophilus* species are among the organisms so implicated [2]. Cultivation of this bacterium has therefore been a goal of clinicians and microbiologists for several decades. Characterization of the WD bacterium at the molecular level was accomplished during the early 1990s, by polymerase chain reaction (PCR) using broad-range primers

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to analyze bacterial 16S rDNA [6, 7]; analysis of the novel sequence established a phylogenetic relationship to the actinomycetes, and the name "*Tropheryma whippelii*" was proposed [7]. In 1997, on the basis of the notion that macrophages are the cell type most prominently involved in the pathology of WD, investigators inoculated interleukin-4-deactivated macrophages with heart-valve tissue affected by WD and reported propagation of bacteria [8]. However, this finding could not be confirmed in subsequent studies [9].

Long-term cocultivation of the WD bacterium with a human fibroblast cell line inoculated with heart-valve tissue was reported by Raoult et al. in 2000 [10]. The infection status of the fibroblasts was determined by microscopy, by periodic-acid-Schiff (PAS) staining, and by immunofluorescence, with the patient's serum. After inoculation of 1 cm² of cell monolayer, the cultures were expanded to 3750 cm² of infected cells over 7 passages within 9 months. After each passage, qualitative PCR detected DNA of the WD bacterium. The estimated bacterial doubling time was 18 days, which is longer than that of any other characterized bacterium. A second strain was subsequently isolated from a duodenal biopsy specimen [11], and the species designation was modified to "*whipplei*" [12]. Taken together, these reports provide good evidence for in vitro propagation of *T. whipplei*. Nonetheless, two important types of data are missing: (1) quantitative assessment of bacterial growth in vitro, by a molecular method, and (2) physical association of the *T. whipplei* 16S rRNA sequence with cultivated bacterial cells, by fluorescent in situ hybridization (FISH). The latter has been proposed as an important link between bacterial sequence and visible cells, especially when new taxa are described [13, 14]. Furthermore, the presence of viable *T. whipplei* bacteria has not been established in the CNS of individuals with WD. The availability of two cerebrospinal fluid (CSF) samples with large numbers of WD bacteria provided an opportunity to isolate new strains of *T. whipplei* and address all of these important issues.

PATIENTS, MATERIALS, AND METHODS

Patients and specimens. This work was approved by the Stanford University Administrative Panel on Human Subjects in Medical Research. CSF from 2 patients was used in these cultivation studies. Both patients presented with intestinal WD that was diagnosed by histopathology and by PCR analysis of *T. whipplei* 16S rDNA. Case 1 was a 74-year-old German man; the CSF specimen was obtained for the purpose of staging, before the initiation of therapy. The patient had no neurological symptoms or signs. Case 2 was a 52-year-old German woman; staging examinations by PCR analysis of *T. whipplei* in CSF [15] revealed CNS infection, but the patient had no neurological symptoms or signs. The patient was treated with an initial

course of 2 weeks of penicillin plus streptomycin, followed by 1 year of oral cotrimoxazole. The CSF specimen used for culture was obtained for the purpose of monitoring response to therapy, 24 months after diagnosis and 12 months after discontinuation of antibiotics. At that time, results of PCR analysis of *T. whipplei* 16S rDNA of duodenal tissue were negative, and histology showed remission, in accordance with published criteria [16]. Diagnostic PCR analysis of *T. whipplei* 16S rDNA [15] showed strongly positive results for the CSF specimens from both patients, and the amplified sequence was completely homologous to the *T. whipplei* 16S rDNA (GenBank accession number X99636).

Cultivation methods. Cell cultivation on human fibroblasts was performed essentially as described elsewhere [10–12], with the following modifications: HEPES buffer (12.5 mM) was used in the medium, and fetal-calf-serum content was reduced from 10% to 1%, after confluent cell monolayers were obtained and before inoculation with bacteria. MRC-5 primary human embryonic lung fibroblasts (CCL-171; American Type Culture Collection) were cultivated in 25-cm² tissue-culture flasks (5-mL medium) and were inoculated with 500 μ L of original CSF. Initial passages of the cultures were performed in 25-cm² flasks; 75-cm² flasks (25 mL) and 150-cm² flasks (35 mL) were later used for large-scale cultures. Each passage of the cultures involved inoculation of 20%–25% of the volume of supernatant onto new fibroblast monolayers after 4–6 weeks of incubation. Medium was changed infrequently: during the first passage, the medium was changed only after 3 weeks, and, during subsequent passages, the medium was either not changed or changed only after ~4 weeks of incubation. Beginning with the 13th passage, both MRC-5 cells and primary human foreskin fibroblasts (a gift from E. S. Mocarski, Stanford University) were used in parallel, for cultivation.

For quantitative measurement of bacterial growth, cell monolayers were cultivated in 6-well tissue-culture plates (9.5 cm²/well) containing 2 mL of medium. On day 0, duplicate wells were inoculated with 0.5 mL of vigorously vortexed culture supernatant from a flask containing infected material. The contents of these wells were harvested on days 1 and 28 after inoculation: first, 1.25 mL of culture supernatant was removed, and then the cell monolayer was removed by a cell scraper and was harvested together with the residual 1.25 mL of supernatant. Both portions were frozen (–80°C) before analysis.

PCR. Tissue-culture supernatant or cell monolayers were centrifuged (18,000 g for 10 min), and DNA from the pellet was extracted as described elsewhere [15, 17]. To detect the presence of *T. whipplei* 16S rDNA, qualitative PCR using primers whip1 and whip2 [17] was performed; for bacterial identification, PCR using broad-range primers 8FPL plus 806R and 515FPL plus 1492RPL to analyze bacterial 16S rDNA [18]. Quantitative competitive PCR was performed according to

published protocols [19] and used the primers whip1 and whip2 [17] and a synthetic internal-standard molecule. This molecule (the "mimic") was constructed by PCR, according to instructions from the Clontech PCR mimic-construction kit. Composite primers were designed on the basis of the sequence of the *Bordetella bronchiseptica* filamentous hemagglutinin gene, *fhaB* [20], and *T. whipplei* 16S rDNA, so that the mimic consisted of a 217-bp sequence including whip1 and whip2 primer sequences at its ends. As a result, the mimic was easily distinguished, on the basis of size, from the 267-bp 16S rDNA amplification product (the "target") of *T. whipplei*. The product from *T. whipplei* and the mimic were each cloned into the TA vector (Invitrogen), plasmid DNA was extracted and quantified, and stock solutions containing 10^8 copies of each plasmid molecule/ μ L were prepared. Serial dilutions of the mimic molecule were used as internal standards in the PCRs, and serial dilutions of the *T. whipplei* product were used as quantitative references in control reactions. Samples from culture were initially tested against 10-fold dilutions of the "mimic," and then, for more accurate measurement, against 2-fold dilutions. The mimic concentration that, in agarose-gel electrophoresis, gave DNA-band intensity equal to that of the *T. whipplei* product was used to estimate the number of copies of *T. whipplei* rDNA in the sample.

Nucleic-acid staining. Nucleic acids in cultivated material were stained directly with YO-PRO-1 fluorescent dye (Molecular Probes). Culture supernatant was fixed in 3.7% formaldehyde, spotted onto glass slides, and air-dried. The slides were then overlaid with 2 μ M YO-PRO-1 in water, incubated for 15 min, rinsed with water, immersed for 15 min in water, rinsed again, air-dried, and mounted with Vectashield mounting fluid (Vector Laboratories) and a coverslip (all steps were performed in the dark).

FISH. FISH was performed essentially as described elsewhere [21], with some modifications. In brief, culture supernatant was centrifuged (10,000 g for 10 min), and the pellets were resuspended in 1 \times PBS, mixed with an equal volume of ethanol (final concentration, 50%), spotted onto Teflon-coated 10-well slides (Erie Scientific), and air-dried, at 45°C, on the wells. The samples on the slides were then fixed by incubations of 3 min each in 50%, 80%, and 96% ethanol. Hybridization was performed for 2 h at 46°C, with a solution containing 5 \times SET, 1% SDS, 10% dextran, 0.2% bovine serum albumin, 0.1 mg polyadenosine/mL, and 5 μ g of labeled probe/mL. The slides were then washed 3 times, for 10 min at 46°C, with 0.2 \times SET at 46°C, rinsed with water, stained with 1 μ M YO-PRO-1 in 1 \times SET as described above, rinsed again, and mounted with Vectashield and coverslips. The following oligonucleotide probes were used: the *T. whipplei*-specific probe Tw16S-652 (5'-TTCCGCTCTCCCCTATCGCACTCT), the negative-control probe Tw16S-Cnt (5'-AAGGCGAGAGGGGATAGCGTGAGA

[21], the broad-range bacterial probe Eub16S-338 (5'-GCTGCC-TCCCGTAGGAGT) [22], and the probe HGC69a (5'-TATAGT-TACCACCGCCGT) for gram-positive bacteria with high G+C content [23]. Tw16S-652, Tw16S-Cnt, and HGC69a were labeled with the fluorophore Cy-3, and probe Eub16S-338 was labeled with Cy-5. Cultures of "*Corynebacterium aquaticum*" (ATCC 14665), *Cellulomonas cellulans* (ATCC 27402), and *Agromyces ramosus* (ATCC 25173)—all *Actinobacteria*—were used as bacterial controls. Slides were viewed and images were recorded by use of a BioRad MRC-1024 Laser Scanning Confocal Imaging System, as described elsewhere [21].

Electron microscopy. Cell monolayers were cultivated on round, 18-mm glass coverslips in 12-well (4-cm²) tissue-culture plates. Four weeks after inoculation, the medium was removed, and the cells were fixed, for 2 days, with 1.5% glutaraldehyde that was buffered to pH 7.3 by sodium cacodylate and that was made isotonic by the addition of sucrose. For scanning electron microscopy (SEM), the coverslips with cells and bacteria were dehydrated with alcohol and a critical-point bomb, were sputter-coated with 100-Å gold, and then were examined by use of an Hitachi S-2400 scanning electron microscope operating at an accelerating voltage of 15 kV. For transmission electron microscopy (TEM), the monolayers were postfixed, for 1 h, in 2% buffered osmic acid, dehydrated with alcohol, and embedded in epoxy resin. Sections were cut at 50-nm thickness, were stained serially with uranyl acetate and lead hydroxide, and then were examined by use of a Phillips 200 electron microscope operating at an accelerating voltage of 75 kV.

Strain deposition. The isolate from patient 2 (strain TW08/27) has been deposited in the American Type Culture Collection (ATCC culture number pending).

RESULTS

Six weeks after inoculation of MRC-5 primary human embryonic lung fibroblast monolayers with CSF from cases 1 and 2, qualitative PCR used to test for *T. whipplei* in culture supernatants from the 2 infected monolayers gave positive results. Cellular and bacterial material from 5 mL of supernatant was then concentrated, by centrifugation, in 1 mL and then was inoculated onto fresh monolayers in 25-cm² flasks. On days 1 and 15 after this passage, 100 μ L of supernatant was collected and analyzed by quantitative PCR. A "low-resolution" quantitative-PCR analysis (using 10-fold dilutions of the mimic) indicated an increase in rDNA copy number, from 10^5 /mL (CSF of case 1) and $<10^5$ /mL (CSF of case 2) on day 1 to $\geq 10^6$ /mL (in both cases) on day 15. Before inoculation, the original CSF specimens had shown copy numbers of 10^4 /mL (case 1) and $<10^4$ /mL (case 2), by the same PCR. In the subsequent, similar passage, supernatant from both cultures was stained with YO-PRO and showed small, rod-shaped bacteria in a characteristic

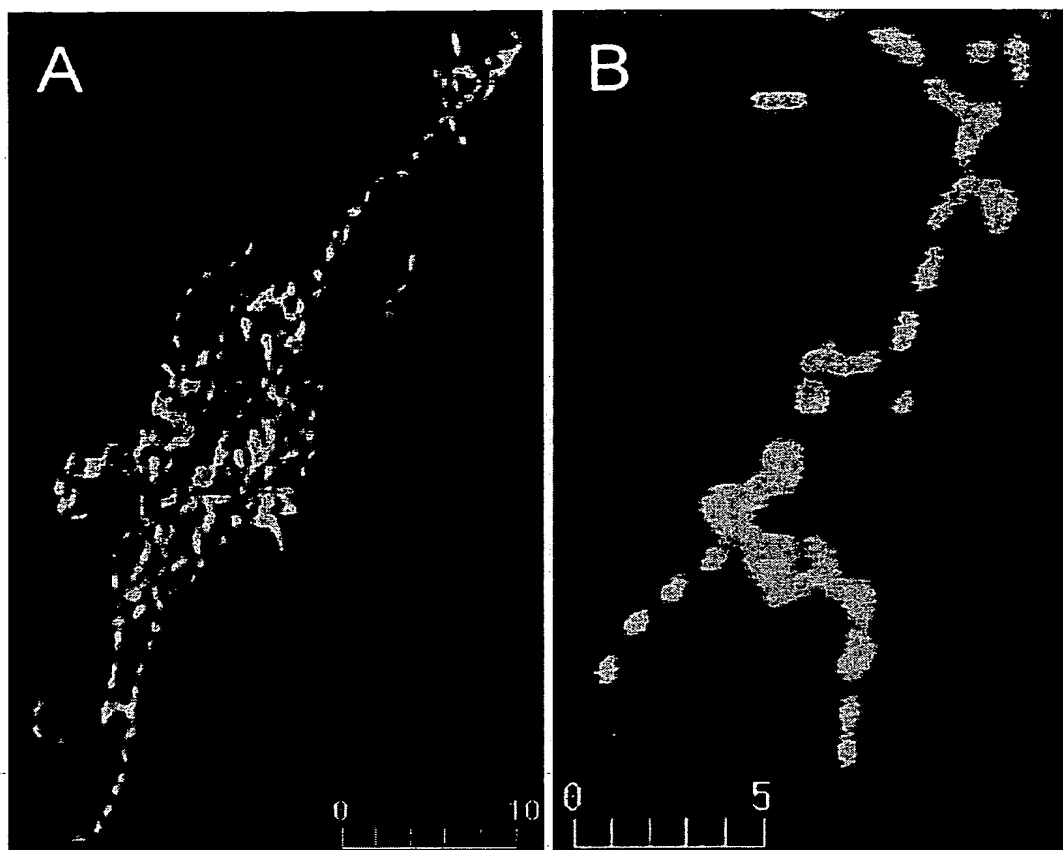


Figure 1. Photomicrograph of culture supernatant stained with YO-PRO nucleic-acids dye. *A*, Strain TW09/02 in third passage (original magnification, $\times 3000$). *B*, Strain TW08/27 in ninth passage (original magnification, $\times 5000$). Scale bars represent micrometers.

chainlike arrangement (figure 1A). Culture supernatant was also examined by PCR using broad-range primers to analyze bacterial 16S rDNA, in an assay that targets a 1443-bp region of the 16S rDNA. Direct sequencing of PCR products revealed unambiguous readings; the sequence from case 1 was a perfect match to that of *T. whipplei* [24]; the products from case 2 had only 2 nucleotide mismatches, in positions where they would not affect the 16S rRNA structure.

A total of 15 passages were performed with both cultures, over a period of 17 months. Beginning with the 13th passage, human foreskin fibroblasts were used in parallel with MRC-5 cells, because they appeared to form more-coherent monolayers and remained morphologically unaltered over longer incubation times. Cultures were regularly checked for the presence of bacteria, by staining the supernatant with YO-PRO; this was done at each passage, usually between the fourth week of incubation and the time of transfer to a new cell monolayer, and showed characteristic-looking bacteria (figure 1B). The strain from case 1 was designated "TW09/02," the strain from case

2 "TW08/27." The cultures were expanded to 40 flasks (150 cm² each) for strain TW09/02 and to 60 flasks for strain TW08/27. PCR analysis of broad-range bacterial 16S rDNA was repeated with both strains after their 15th passage, with the same results. Material from the 60 flasks with strain TW08/27 was harvested, and bacterial DNA was extracted and used for a genome-sequencing project [25] (see the http://www.sanger.ac.uk/Projects/T_whipplei/ Web site).

Quantitative-PCR studies (see Patients, Materials, and Methods) were performed again after the 11th passage, using 10-fold and, subsequently, 2-fold dilutions of the mimic (figure 2). Data from supernatants and data from combined fractions (supernatant plus cell monolayer) harvested on days 1 and 28 after inoculation were compared (table 1). *T. whipplei* 16S rDNA copy numbers were ~ 100 -fold greater on day 28 than they were on day 1. In addition, rDNA copy numbers in the combined fractions were ~ 10 -fold greater than those measured in the supernatants alone. These data suggest that, on average, the bacteria have completed 7 divisions during the intervening

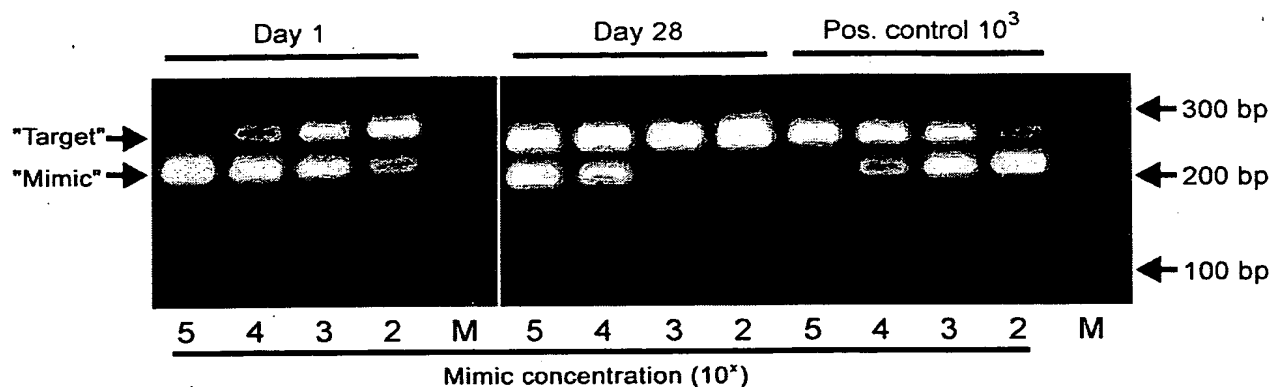


Figure 2. Agarose gel showing results of polymerase chain reaction (PCR) in cultures in 6-well plates (see Patients, Materials, and Methods), for combined fractions (supernatant plus cell monolayer) from strain TW08/27, on days 1 and 28 of incubation, tested against initial 10-fold dilutions of the "mimic" molecule. A positive control with the cloned *Tropheryma whipplei* "target" (10^3 copies) was also used. The copy numbers of mimics and targets used in a 50- μ L PCR are given. Because of dilution factors, the calculated copy number per milliliter of culture material (table 1) is 1.5 log higher than the copy number used in a 50- μ L PCR. M, molecular-weight marker.

27 days, which corresponds to a bacterial generation time of ~4 days. To confirm the specificity of the quantitative-PCR results, a target band from this assay was sequenced, for both strains; this sequence was identical to the 16S rRNA sequence of *T. whipplei*.

For both strains, FISH experiments with culture supernatant were performed after the 12th and 15th passages. All visible bacteria in supernatants hybridized with the *T. whipplei*-specific probe Tw16S-652, the broad-range bacterial probe Eub16S-338, and the actinobacterial probe HGC69a but not with the negative-control probe Tw16S-Cnt. All bacterial control strains hybridized with Eub16S-338 and HGC69a, none hybridized with Tw16S-Cnt, and only "*C. aquaticum*" hybridized, very faintly, with Tw16S-652, as described elsewhere [21]; this faint signal was easily distinguishable from the much-brighter signal in the 2 CSF cultures. Triple-label experiments, with YO-PRO, Tw16S-652, and Eub16S-338, revealed colocalized staining patterns with the 3 labels, for all bacteria in both cultures (figure 3), indicating a homogenous population of (*T. whipplei*) bacteria.

Electron microscopy of culture material from both strains was performed after the 14th passage. SEM showed intact extracellular bacteria (figure 4A), and TEM showed well-preserved bacteria both in extracellular locations and within the cytoplasm of healthy-appearing fibroblasts (figure 4B).

DISCUSSION

The results of the present study indicate that viable *T. whipplei* strains are found in the CSF of patients with WD and that they can be propagated in the presence of human fibroblasts in culture. These data confirm and expand on the findings reported by Raoult et al. [10–12]. They also provide the first

quantitative measurement of the growth of *T. whipplei* in vitro. A previous report [8], describing the growth of *T. whipplei* in interleukin-4-deactivated macrophages, has been not confirmed, either by us (M.M. and D.A.R., unpublished results) or by other investigators [9].

Our data also document the first cultivation of *T. whipplei* from CSF samples. CSF is ideally suited for such studies, since it is a relatively simple fluid that is normally sterile. The examination of CSF has special relevance for diagnostic testing for WD, because (1) bacteria appear to invade the CNS early in the disease and (2) late manifestations affecting the CNS pose a significant threat to patients [2, 15]. This is illustrated by a number of published cases with symptomatic CNS disease, cases in which bacteria appeared to have been eradicated from the intestinal mucosa after therapy [2, 15, 17, 26–29]. One noteworthy case presented with severe insomnia as the only symptom 8 years after intestinal WD had been diagnosed and treated; at that time, results of intestinal/histological examination and PCR analysis of intestinal tissue were negative but PCR analysis showed that CSF was positive for *T. whipplei* [29].

Table 1. Results of quantitative polymerase chain reaction, for the 2 *Tropheryma whipplei* strains from cultures in 6-well plates (see Patients, Materials, and Methods).

Day after inoculation	Strain TW09/02		Strain TW08/27	
	Supernatant	Combined fraction	Supernatant	Combined fraction
1	5×10^3 /mL	5×10^4 /mL	$<5 \times 10^3$ /mL	2×10^4 /mL
28	8×10^5 /mL	8×10^6 /mL	5×10^5 /mL	5×10^6 /mL

NOTE. Both the culture supernatants (1.25 mL) and the combined fractions (1.25 mL), the latter of which consisted of supernatant plus cell monolayer, were tested during the 11th passage on each of the 2 days.

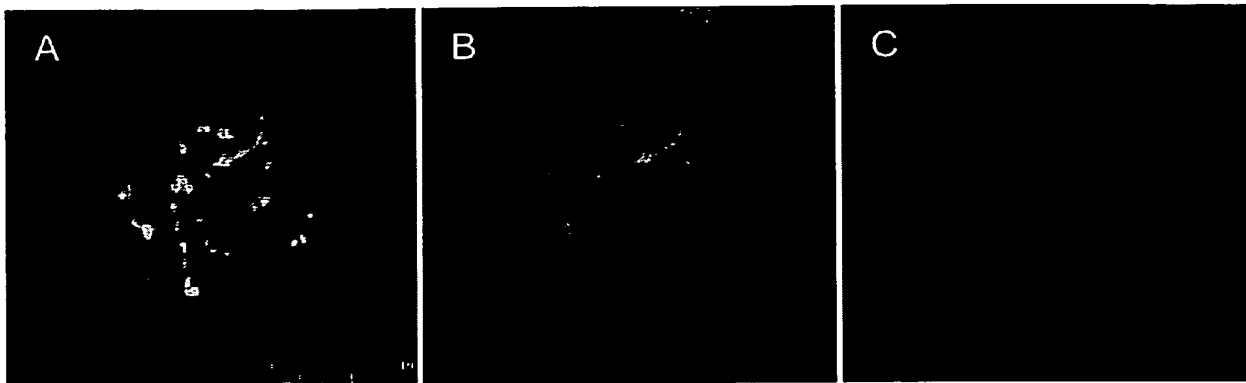


Figure 3. Photomicrographs after fluorescent in situ hybridization with strain TW08/27 from the 14th passage of the cultures, for dual hybridization with probes Tw16S-652 and Eub16S-338, followed by YO-PRO stain. A, YO-PRO stain (nonspecific DNA stain) viewed with the fluorescein isothiocyanate channel. B, Probe Tw16S-652 (*Tropheryma whipplei* specific) viewed with the Texas Red channel. C, Probe Eub16S-338 (bacterial broad range) viewed with the Cy-5 channel (original magnification, $\times 2000$). Scale bar represents micrometers.

Often, manifestations of *T. whipplei* in CNS respond only partially to antibiotics and have a poor prognosis.

A previous study examined CSF samples from 24 patients with WD that were obtained at various times before and after therapy [15]; even in neurologically asymptomatic patients, PCR results were positive for the presence of *T. whipplei* in 7 of 10 cases before therapy and in 3 of 11 cases after therapy. These data indicate that the bacterium or its components are commonly present in the CNS of patients with intestinal WD and that, even in the presence of prolonged therapy with antibiotics, bacterial clearance may be delayed or uncertain. Furthermore, the data underscore the importance of using antibiotics that cross the blood-brain barrier. The isolation of 2 *T. whipplei* strains from CSF supports these concepts and emphasizes the importance of PCR-based, sensitive approaches for the detection and monitoring of CNS infection. The present study provides new evidence of viable *T. whipplei* in the CNS of patients with WD, even in the absence of neurological symptoms, and demonstrates that the bacterium can persist in a viable state, even after 1 year of therapy and intestinal-disease remission.

Quantitative measurement of bacterial growth is an important contribution to the evolving story of the propagation of *T. whipplei* ex vivo. The use of an internal standard (i.e., a mimic) avoids the potential problems of other types of PCR assays, in which PCR inhibitors might interfere with quantification [19]. Our calculated doubling time of 4 days differs from the previously reported time of 18 days, which was based on semiquantitative microscopic assessment of inclusions in fibroblast monolayers, inclusions that were shown to be positive for *T. whipplei* [10] when the PAS reagent was used, but it is still among the longest observed doubling times for any bacteria. This difference might be due either to the different mea-

surement methods or culture conditions or to the differences between *T. whipplei* strains. Knowledge of the generation time is clinically relevant; with a doubling time of 4 days, a typical, 14-d intravenous therapy-induction period [30] spans only 3 replication cycles and thus might have to be reconsidered.

Bacterial morphology and the chainlike arrangement were distinctive when revealed by YO-PRO staining (figure 1). FISH now integrates, for the first time, bacterial morphology and the 16S rRNA sequence of *T. whipplei*. A previous study with sections from intestinal biopsy specimens did not resolve individual bacteria, probably because of high bacterial density and the thickness of the sections [21]. Triple-label experiments in the present study (figure 3) showed that nonspecific staining of DNA by YO-PRO, a broad-range bacterial probe, and a WD-specific probe all colocalized to the same bacterial shapes. These data and the absence of ambiguities in the PCR-based analysis of broad-range bacterial 16S rDNA performed during the third and 15th passages indicate that the cultures were not contaminated with other bacteria. Multiple FISH experiments clearly showed small, rod-shaped bacteria, but the slender shapes and the chainlike arrangement were not as well preserved as were those seen in staining by YO-PRO. The different morphologies seen by these 2 methods may arise from the different fixation procedures (i.e., formalin vs. alcohol) and/or the additional processing steps employed in the FISH protocol.

Uncertainty remains as to whether *T. whipplei* prefers intra- or extracellular growth environments. A detailed electron-microscopic study of intestinal WD [31] demonstrated that the majority of morphologically intact bacteria were located extracellularly in the lamina propria and that intracellular bacteria were in various stages of degradation. These findings are consistent with the results of more-recent work, which used FISH in intestinal biopsies [21] and which found *T. whipplei*-rRNA

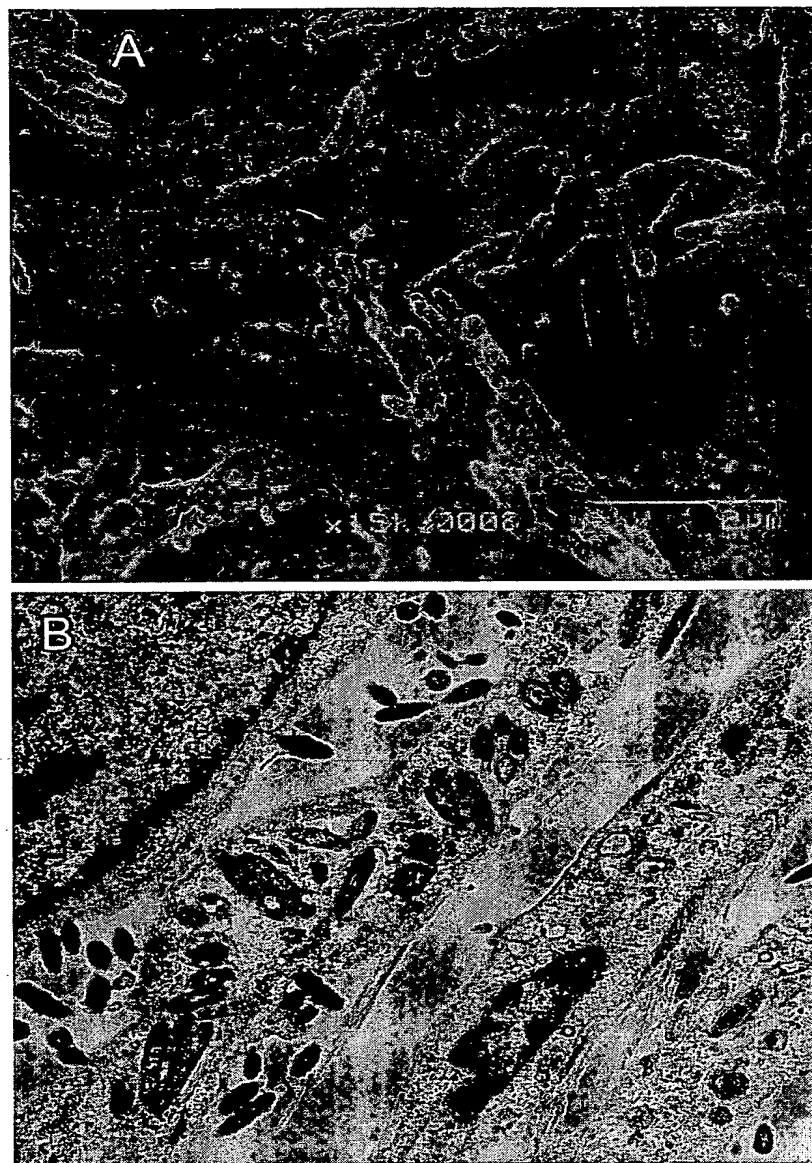


Figure 4. Electron micrographs of *Tropheryma whippelii* in fibroblast cell culture after the 14th passage. *A*, Results of scanning electron microscopy of strain TW08/27 (original magnification, $\times 20,000$). *B*, Results of transmission electron microscopy of strain TW09/04 (original magnification, $\times 12,275$).

hybridization signals, corresponding to metabolically active bacteria, in the lamina propria, directly subjacent to the epithelial basement membrane, but not inside cells. The location of the rRNA signal did not correspond to the inclusions characteristic of macrophages from patients with WD, inclusions that PAS shows to be positive for *T. whippelii*. On the other hand, Raoult et al. [10] reported intracellular growth in their fibroblast cell-culture system, which used PAS and immunofluorescence staining. In the present study, quantitative PCR

with supernatant and with combined fractions indicated that *T. whippelii* grows in close association with fibroblasts but also grows in the cell-free supernatant. SEM clearly showed bacteria in extracellular locations (figure 4A); on the other hand, TEM showed intact bacteria in both intra- and extracellular locations (figure 4B). The host cells too appeared to be intact, and this obvious lack of cell damage is reminiscent of the paucity, in *T. whippelii* infection in humans, of both cell damage and inflammatory cellular infiltrate [16].

The *T. whippelii* isolate TW08/27 has been subjected to complete-genome sequencing [25] (see the http://www.sanger.ac.uk/Projects/T_whippelii/ Web site). Among fastidious and cultivation-resistant bacterial pathogens, the genome of *T. whippelii* is the third to be sequenced, after those of *Treponema pallidum* and *Mycobacterium leprae*. Although resistance to cultivation is uncommon among known pathogenic bacteria, the vast majority of bacteria in natural environments and in the commensal flora have not been cultivated in vitro [32, 33]. The extent to which currently uncharacterized or uncultivated bacteria might be involved in chronic idiopathic diseases is unclear [34]. In this context, *T. whippelii* is an attractive model organism with which to study such questions and, thus, to gather insight into related, important biological principles.

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